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Protein and isozyme analysis in different species, varieties and populations of *Tabernaemontana*

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Abstract
Tabernaemontana is a member of Apocynaceae. Approximately 100 species of this genus are widely distributed in tropical parts of the world. Phytochemical studies revealed that in *T. coronaria* 66 different alkaloids have been identified from different parts. 22 types of alkaloids have been isolated from root and bark of *T. dichotoma*. The present investigation includes total protein analysis and study of isozyme activities showing distinct variations. Total protein analysis, both quatitative and qualitative, showed significant variations. Two isozymes indicated variations at species and variety levels. Proteins and isozymes are significant and widely used to study genetic diversity at interspecific and intraspecific levels. The number and position of isozyme bands differed in two species of *Tabernaemontana* and three varieties of *T. coronaria*. The differential distribution of both esterase and peroxidase activities has revealed genomic diversity among the species and varieties of *Tabernaemontana*. The differences among the populations are very low indicating their vegetative propagation.

Keywords: dendogram, isozyme, protein, *Tabernaemontana*

Introduction

Tabernaemontana (synonym- *Ervatamia*) belongs to the family Apocynaceae, subfamily Plumeroidae and tribe Tabermontanae. An approximately 100 species of *Tabernaemontana* are widely distributed in tropical countries in wild condition including India as a garden plant. The plant is medicinally important with anti-ulcer, anti-bacterial and anti-inflammatory properties and is also used as antihelmintic, antihypertensive, diuretic, hair growth promoter, purgative and many other illnesses [1, 2]. This genus is important as a natural synthesizer of different alkaloids including many indole alkaloids. Phytochemical studies on various parts of this plant reveal that it contains at least 66 indole alkaloids, non- alkaloid constituents like enzymes, flavonoids, hydrocarbons, phenolic acids, phenyl propanoids, steroids and terpenoids. The bark and leaf of *T. dichotoma* are purgative. *T dichotoma* is used in healing of wounds caused by snake bites and bites of centipedes [3].

The species, varieties and populations of *Tabernaemontana* considered in the present investigation reflected their individuality by their morphological differences [4, 5] (Fig. 1). The somatic chromosome number was found to be 2n =2x = 22 chromosomes in *T. dichotoma* [6, 7, 8, 9] and other varieties and populations of *T. coronaria* except *T. coronaria* var. *florepleno* where it was 3n = 3x = 33 chromosomes [10]. Karyotypic details revealed cryptic structural alteration of chromosomes that led to the possession of distinct different karyopypes in each species and varieties.

The growth and development of plant cells involve changes in different biochemical contents bringing about diversification and specialized characteristics of different multicellular organs. With the progression of cellular differentiation there is continuous synthesis of and/ or degradation of specific structural proteins and enzymes which results in morphological and anatomical development and functional specialization of a particular tissue as well [11]. Both qualitative and qualitative changes in various proteins may occur during maturation of cells.

The species and populations of a particular plant can be characterized by their protein profile and specific isozyme activities at specific period of growth and development. In protein analysis polyacrylamide gel electrophoresis (PAGE) is an important and useful analytical tool for separation and quantification of specific polypeptides. It is a method of choice for locating any qualitative changes in protein metabolism at the cellular or tissue level [12, 13, 14]. The unique advantage of this process is that a mixture of polypeptides can be separated and visualized permitting estimation of different polypeptide contents. The PAGE technique has been successfully utilized for separation of both plant proteins and isozymes from both *in vivo* and *in vitro* conditions [15, 16, 17].

The isozymes played a significant role in plant biochemistry research when genetic polymorphisms for isozymes within the same populations were observed [18]. Isozymes have proven to be reliable marker for studying systematics, genetics, breeding and interrelationship of a group of plants. The activity of isozymes has also been utilized as a suitable marker during plant cell growth and development *in vitro* [19, 20]. There are different isozymes found in plant system of which esterase and peroxidase are used to identify different cultivars including somaclones developed against biotic and abiotic stress tolerance. Esterase belonging to the class hydrolase, involves esterification. Peroxidase, on the other hand, accelerates the reaction of hydrogen peroxide, a toxic metabolic product of cell, to form water and oxygen. In absence of peroxidase this reaction occurs spontaneously with slow rate.

The present biochemical investigation was undertaken to find

the variations or changes, if any, as well to study interrelationship in the quantitative and qualitative protein profiles as well as isozyme activity patterns of esterase and peroxidase among different species, varieties and populations of *Tabernaemontana*.

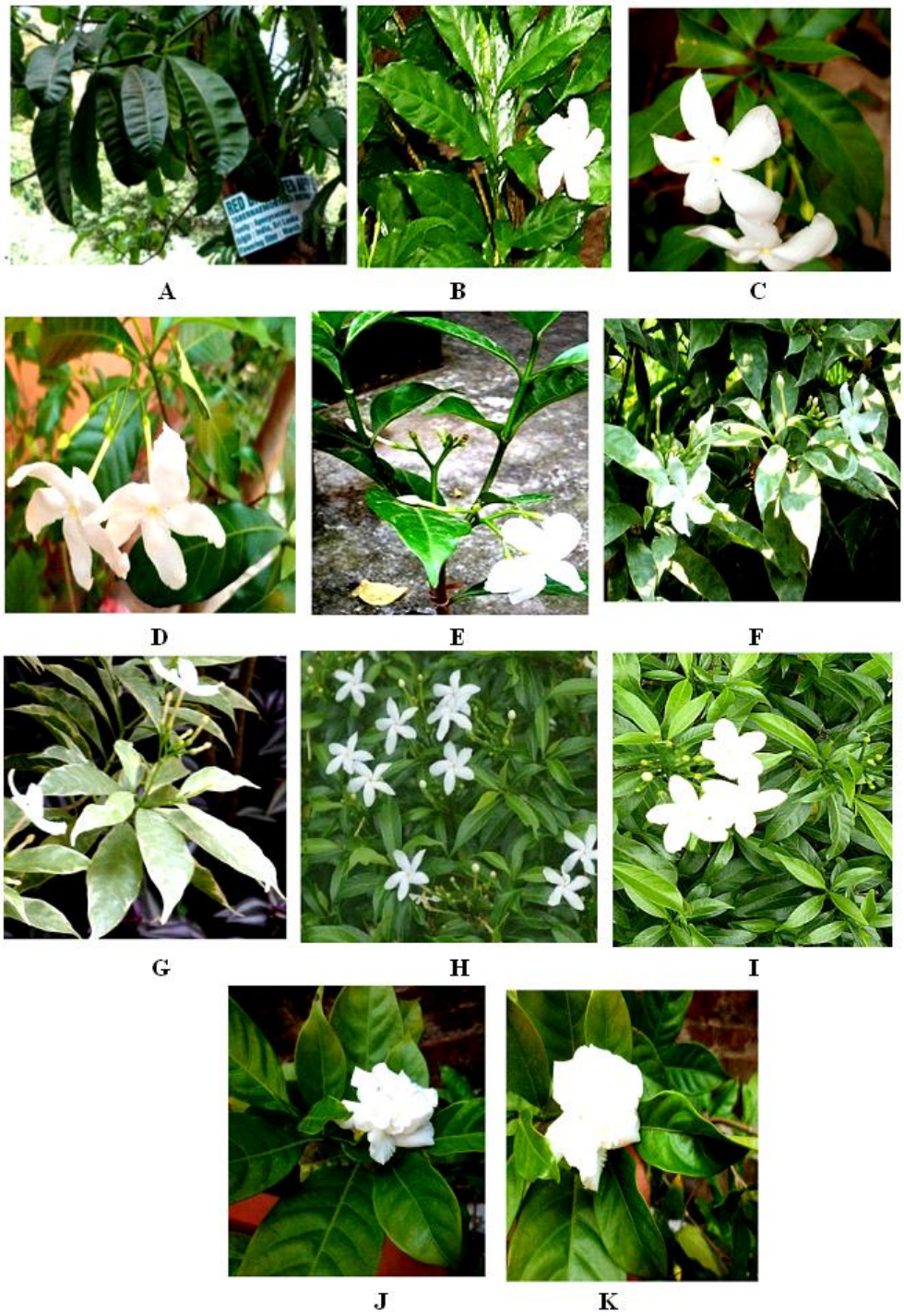

Fig 1: A= *T. dichotoma* (PI), B-E= *T. coronaria* (PI-PIV), F-G= *T. coronaria* var. *variegata* (PI, II), H-I= *T. coronaria* var. *dwarf* (PI, II) and J-K= *T. coronaria* var. *florepleno* (PI, II)

Materials and methods

Plant materials

In the present study 11 plants were used for biochemical investigations. These are *Tabernaemontana dichotoma* Roxb. ex Wall. (Population I), *T. coronaria* (Jacq.) Willd. (Population I-IV), *T. coronaria* var. *variegata* (Population I-II), *T. coronaria* var. *dwarf* (Population I-II), and *T. coronaria* var. *florepleno* (Population I-II). Plants were collected from different populations of West Bengal and were identified by the experts of Central National Herbarium, Howrah. The list of collected plant is represented in Table 1.

Table 1: Collection details and morphological characters of different species and varieties of *Tabernaemontana*

Name of the plant	Characters
Tabernaemontana dichotoma Roxb. ex Wall. (Population I- Kolkata)	Perennial, tree; leaf broad lanceolate; inflorescence cymose; flower star shaped, petals present in single whorl; fruit follicle.
T. coronaria (Jacq.) Willd. (Population I- Howrah, population II- Kolkata, population III- Hooghly and population IV- Midnapore)	Perennial, evergreen shrub; leaf elliptical; inflorescence cymose; flower salver shaped; petals present in single whorl.
T. coronaria var. *variegata* Hort. (Population I- Howrah and population II- Kolkata)	Perennial, medium sized shrub; leaf variegated; inflorescence cymose; flower salver shaped; petals present in single whorl.
T. coronaria (Jacq.) Willd.var. *dwarf* (Population I- Howrah and population II- Kolkata)	Perennial, small, compact; leaf small; inflorescence cymose; flower salver shaped; round petals present in single whorl.
T. coronaria (Jacq.) Willd. var. *florepleno* (Population I- Howrah and population II- Kolkata)	Perennial, evergreen large plant; leaf large, thick and darker; inflorescence cymose; flower large; petals present in double whorls.

Methods

Isolation of total protein: Protein was extracted from young leaves. Leaves were crushed with Protein Extraction Buffer (0.1 M Tris base, 0.25 M sucrose, 1% PVP, 0.1% ascorbic acid, 0.1% cystein HCl, 1 mM sodium EDTA and 1 mM magnesium chloride, pH 6.8) and centrifuged at 15,000 rpm for 20 minutes at 4ºC.

Quantitative estimation of total protein

Quantitative estimation was done according to Bradford method [21] using a spectrophotometer at 595 nm. The amount of protein was plotted against the absorbance in a standard curve to determine the unknown sample.

Qualitative estimation of total protein

Qualitative estimation was carried out by SDS-PAGE using 12% resolving gel and 5% stacking gel. Sample extracts protein was mixed with equal volume of sample loading buffer containing 2% SDS, 10% glycerol, 1 M Tris HCl (pH 6.8) and 0.1% bromophenol blue [22] and after boiling loaded into wells. Electrophoresis was performed at a constant voltage of 70V. After electrophoresis, the gel was immersed in 0.1% Coomassie Brilliant Blue for overnight followed by destaining till clear bands resolve optimally. Protein ladder was used as marker.

Study of isozyme activity

Isozyme activity was measured from young leaves. After isolation of protein, enzyme activity was measured by NATIVE-PAGE using 8% resolving gel and 5% stacking gel. Sample extracts containing protein was mixed with equal volume of sample loading buffer and loaded into wells. For esterase activity after electrophoresis gel was immersed in sodium phosphate buffer containing 0.01% α-NAA and 0.01% β-NAA till bands resolve optimally and for peroxidase activity the gel was immersed in sodium phosphate buffer containing hydrogen peroxide and guaiacol (Sigma) solution.

Molecular Weight Determination

This is done by SDS-PAGE of proteins of known molecular weight along with the protein to be characterized. A linear relationship exists between the logarithm of the molecular weight of an SDS-denatured polypeptide or native nucleic acid and it's Rf (relative flow). The Rf is calculated as the ratio of the distance migrated by molecule to that migrated by a marker dye-front. A simple way of determining relative molecular weight (Rm) by electrophoresis is to plot a standard curve of distance migrated vs. log 10 MW for known samples and read off the log Rm of the sample after measuring distance migrated on the same gel.

Data analysis

After electrophoresis, the stained gel was taken for image formation in a gel documentation system (Viber Lourmat) and analyzed in a computer with Ptotocap MW software to detect the bands. For all the species, varieties and population, bands on protein gels were scored as (1) when present and (0) when absent and a percent of disagreement value distance matrix was formed. This matrix was subjected to UPGMA (unweighted pair group method with arithmetic mean) cluster analysis using joining tree algorithm of Statistica version 5.1 software to generate a dendogram.

Results and discussion

Quantitative estimation of total protein

The total protein content in leaf tissue sample was quantified by Bradford's method which revealed differences in protein contents among species and varieties studied. The lowest total protein content was noted in *T. coronaria* var. *dwarf* population I (6.80 mg/ g) and the highest content was observed in *T. dichotoma* (10.00 mg/ g) (Table 2). Bradford's method of protein quantification was found to be very sensitive, accurate and highly reproducible as well. This dye binding assay is approximately four times sensitive than other available methods.

Qualitative estimation of total protein

Total protein profile clearly revealed differences in the number of bands ranging from 9 in *T. coronaria* var. *variegata* and *T. coronaria* var. *dwarf* to 11 in *T. coronaria* and *T. dichotoma* (Fig. 2). Moreover, the range of protein size varied to a great extent among the species and varieties ($p > 0.5$) of *Tabernaemontana* though the differences were not remarkable at the population level ($p < 0.5$). The largest polypeptide size was observed in *T. coronaria* var. *florepleno* and the lowest polypeptide size was observed in *T. coronaria*. The qualitative differences as observed in buffer soluble total protein profile clearly indicated distinct variations among different species and varieties of *Tabernaemontana*. The polymorphism observed among these species and varieties suggested differences in genomic constitution at its functional level as the tissues were collected in the same period during their maximum growth in the field.

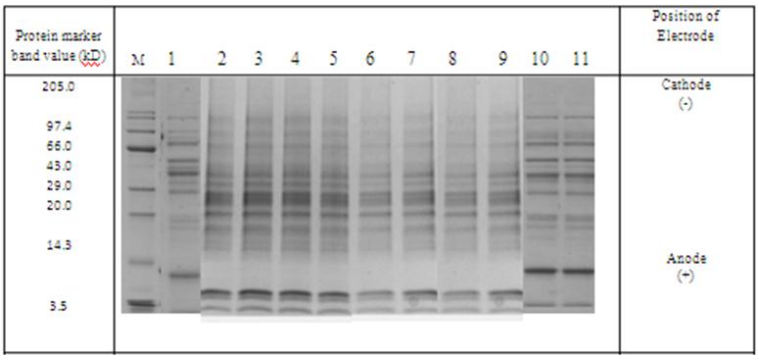

Fig 2: Protein profile in different species, varieties and populations of *Tabernaemontana*. Lane 1= *T. dichotoma* (P I), Lane 2-5= *T. coronaria* (P I-P IV), Lane 6 & 7- *T. coronaria* var. *dwarf* (P I-P II), Lane 8 & 9- *T. coronaria* var. *florepleno* (P I-P II) and Lane 10 & 11- *T. coronaria* var. *variegata* (P I-P II). Lane M= Protein Molecular Weight Marker gave the bands of 205.0 kD, 97.4 kD, 66.0 kD, 43.0 kD, 29.0 kD, 20.0 kD, 14.3 kD and 3.5 kD respectively

Study of isozyme activity

The isozyme activity was determined by the presence of distinct bands in NATIVE gel that contained no charges. The isozyme pattern of esterase activity has clearly revealed the distribution of isoforms in three distinct zones; Zone A- cathode, Zone B- Intermediate and Zone C- Anode zone (Fig. 3). The enzyme esterase has the property in breaking down the ester bonds to produce organic acid and alcohol. In all the species and varieties studied in the present investigation, the number and position of the isozyme activity band differed. At population level, however, no variation was observed in number and position of isozyme band. In *T. coronaria* var. *dwarf*, no band observed in zone B and, on the other hand, no band was also observed in zone C in case of *T. coronaria* var. *florepleno.* (Table 3) Such differential distribution of esterase activity has clearly revealed genomic diversity to certain extent among the species and varieties of *Tabernaemontana*. Among these species, varieties and populations maximum differences in isoforms of esterase were observed in zone B than the other two zones. In general, the esterase activity has been found to be enhanced during plant growth and development and as a result the presence of different zones of activity might suggest that these species and varieties differ in genetic constitutions as reflected in their isozyme activity growth pattern. There are also previous reports on using esterase activity as a genetic marker for genetic polymorphism evaluation in other plant crops [23].

Unlike esterase activity, the peroxidase isozyme activity has only been shown to be distributed in two different zones; Zone A- Cathode Zone and Zone B- Intermediate zone (Fig. 4). No band has been observed in anode zone. In *T. dichotoma* and *T. coronaria* var. *florepleno* peroxidase bands are only present only in cathode zone. Moreover, no variation has been observed in number and position of isozyme bands at population level (Table 3). The differential distribution of bands in esterase and peroxidase activity indicated the genomic differences in different species and varieties of *Tabernaemontana* and therefore, the isozyme pattern may be considered as a unique genetic marker for identification as well as a parameter for physiological changes during growth and differentiation. Isozyme polymorphism has been widely used in identifying horticultural crops [24] and in genetics and plant breeding. These markers are stable and useful in population study [25]. The presence of low level of polymorphisms at population level may be attributed to their vegetative propagation.

Allelic frequency for esterase activity varied from 0.30 to 0.60 (Table 4) and locus 4 and 10 showed highest and lowest % of polymorphism respectively. Allelic frequency for peroxidase activity varied from 0.33 to 0.50 (Table 5) and locus 3 showed highest % of polymorphism and locus 4 and 6 least.

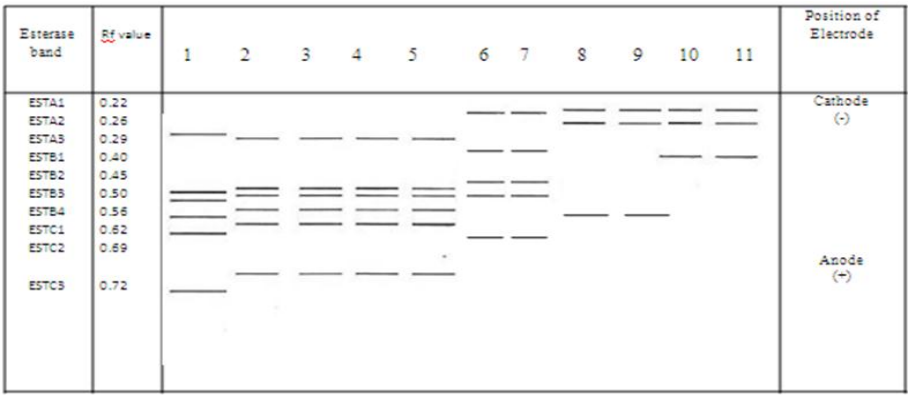

Fig 3: Zymogram of esterase profile in different species, varieties and populations of *Tabernaemontana*.
Lane 1= *T. dichotoma* (P I), Lane 2-5 *T. coronaria* (P I-IV), Lane 6 & 7- *T. coronaria* var. *variegata* (P I-P II), Lane 8 & 9- *T. coronaria* var. *dwarf* (P I-P II) and Lane 10 & 11- *T. coronaria* var. *florepleno* (P I-P II)

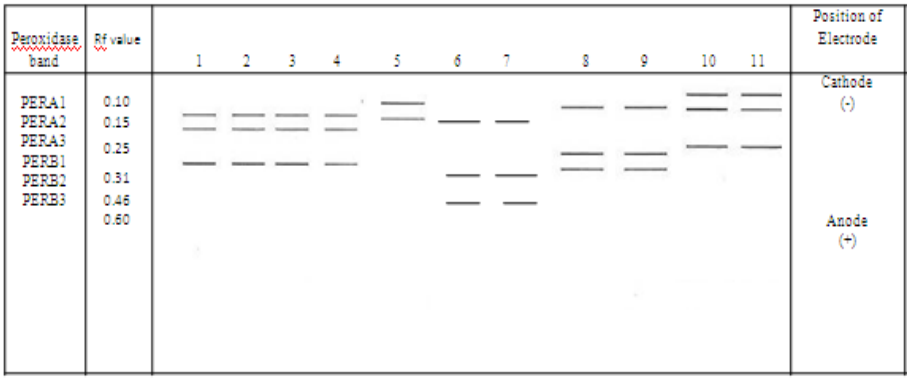

Fig 4: Zymogram of peroxidase profile in different species, varieties and populations of *Tabernaemontana*.
Lane 1-4= *T. coronaria* (P I-P IV), Lane 5= *T. dichotoma* (P I), Lane 6 & 7- *T. coronaria* var. *dwarf* (P I-P II), Lane 8 & 9- *T. coronaria* var. *florepleno* (P I-P II) and Lane 10 & 11- *T. coronaria* var. *variegata* (P I-P II).

Data analysis

Genetic dissimilarities among all the species ranged from 2.00 PDV to 3.74 PDV. The two plants *T. coronaria* and *T. coronaria* var. *variegata* are closely related. The distance between *T. coronaria* population I and *T. coronaria* var. *variegata* population I is 2.00. The distance between *T. coronaria* var. *dwarf* and *T. dichotoma* with *T. coronaria* 3.00 are and 3.46 respectively whereas distance between *T. coronaria* and *T. coronaria* var *florepleno* is 3.74. Between two triploid populations, the distance remains 2.00 PDV (Table 6). UPGMA dendogram using protein and isozyme shows 4 clusters:- cluster 1 consists of 2 populations of *Tabernaemontana coronaria* var. *florepleno*. *T. dichotoma* is present in cluster 2, cluster 3 consists of 2 populations of *T. coronaria* var. *dwarf*, cluster 4 is divided into 2 subclusters:- subcluster 1 consists of 2 populations of *T. coronaria* var. *variegata* and 4 populations of *T. coronaria* are present in subcluster 2. *T. coronaria* var. *florepleno* being triploid is distantly related from other diploid (Fig. 5).

Table 2: Total protein analysis in species varieties and populations of *Tabernaemontana*

Plant	Total protein content (mg/g FW)	Total no. of Protein bands	Range of M.W. (kD)
T. dichotoma P I	10.00	11	6.221-88.844
T. coronaria P I	8.48	11	6.221-80.571
T. coronaria P II	8.50	11	6.221-80.571
T. coronaria P III	8.32	11	6.221-80.571
T. coronaria P IV	8.40	11	6.221-80.571
T. coronaria var. *variegata* P I	7.14	9	6.221-88.844
T. coronaria var. *variegata* P II	7.09	9	6.221-88.844
T. coronaria var. *dwarf* P I	6.80	9	6.221-88.844
T. coronaria var. *dwarf* P II	6.82	9	6.221-88.844
T. coronaria var. *florepleno* P I	9.88	10	13.431-92.007
T. coronaria var. *florepleno* P II	9.72	10	13.431-92.007

P= Population, I-IV= Population number

Table 3: Esterase and peroxidase activity in species, varieties and populations of *Tabernaemontana*

Plant	Total number of esterase bands			Total number of peroxidase bands		
	A zone	B zone	C zone	A zone	B zone	C zone
T. dichotoma P I	1	2	3	2	-	-
T. coronaria P I	1	4	1	2	1	-
T. coronaria P II	1	4	1	2	1	-
T. coronaria P III	1	4	1	2	1	-
T. coronaria P IV	1	4	1	2	1	-
T. coronaria var. *variegata* P I	1	3	1	2	1	-
T. coronaria var. *variegata* P II	1	3	1	2	1	-
T. coronaria var. *dwarf* P I	2	-	1	2	1	-
T. coronaria var. *dwarf* P II	2	-	1	2	1	-
T. coronaria var. *florepleno* P I	2	1	-	2	-	
T. coronaria var. *florepleno* P II	2	1	-	2	-	

A Zone = Cathode zone and B Zone = Intermediate zone, - = band absent

Table 4: Calculation of allelic frequency and % of polymorphic loci from esterase analysis in species, varieties and populations of *Tabernaemontana*

Enzyme activity	Locus no	TD	TC I	TC II	TC III	TC IV	TCV I	TCV II	TCD I	TCD II	TCF I	TCF II	% OF Polymorphic LOCI
Esterase	1	0	0	0	0	0	1	1	1	1	1	1	54.54
	2	0	0	0	0	0	0	0	1	1	1	1	36.36
	3	1	1	1	1	1	0	0	0	0	0	0	45.45
	4	1	1	1	1	1	1	1	0	0	1	1	81.81
	5	0	1	1	1	1	1	1	0	0	0	0	54.54
	6	1	1	1	1	1	1	1	0	0	0	0	63.63
	7	0	1	1	1	1	0	0	0	0	0	0	36.36
	8	1	1	1	1	1	0	0	1	1	0	0	63.63
	9	1	0	0	0	0	1	1	0	0	0	0	27.27
	10	1	0	0	0	0	0	0	0	0	0	0	09.09
	Allelic frequency	0.60	0.60	0.60	0.60	0.60	0.50	0.50	0.30	0.30	0.30	0.30	

TD = *T. dichotoma*, TC = *T. coronaria*, TCV = *T. coronaria* var. *variegata*, TCD = *T. coronaria* var. *dwarf*, TCF = *T. coronaria* var. *florepleno*, I-IV = Population number

Table 5: Calculation of allelic frequency and % of polymorphic loci from peroxidase analysis in species, varieties and populations of *Tabernaemontana*

Enzyme activity	Locus no	TD	TC I	TC II	TC III	TC IV	TCV I	TCV II	TCD I	TCD II	TCF I	TCF II	% OF Polymorphic LOCI
Peroxidase	1	1	0	0	0	0	1	1	0	0	0	0	27.27
	2	0	1	1	1	1	0	0	1	1	1	1	72.72
	3	1	1	1	1	1	1	1	0	0	1	1	81.81
	4	0	0	0	0	0	0	0	1	1	0	0	18.18
	5	0	1	1	1	1	1	1	0	0	0	0	54.54
	6	0	0	0	0	0	0	0	1	1	0	0	18.18
	Allelic frequency	0.33	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.33	0.33	

TD = *T. dichotoma*, TC = *T. coronaria*, TCV = *T. coronaria* var. *variegata*, TCD = *T. coronaria* var. *dwarf*, TCF = *T. coronaria* var. *florepleno*, I-IV = Population number

Table 6: Euclidean distances of total protein and isozyme analysis in species, varieties and populations of *Tabernaemontana*

	A-I	A-II	A-III	A-IV	B-I	B-II	C-I	C-II	D-I	D-II	E-I
A-I	0.00										
A-II	2.00	0.00									
A-III	2.00	0.00	0.00								
A-IV	2.00	0.00	0.00	0.00							
B-I	2.00	2.83	2.83	2.83	0.00						
B-II	2.83	3.46	3.46	3.46	2.00	0.00					
C-I	2.83	2.00	2.00	2.00	2.83	2.83	0.00				
C-II	2.83	2.83	2.83	2.83	2.83	2.83	2.00	0.00			
D-I	3.74	3.74	3.74	3.74	3.74	3.16	3.74	3.74	0.00		
D-II	3.16	3.16	3.16	3.16	3.16	2.45	3.16	3.74	2.00	0.00	
E-I	3.46	3.46	3.46	3.46	3.46	3.46	3.46	2.83	3.16	3.74	0.00

A (I-IV) = *T. coronaria* (PI-PIV); B (I, II) = *T. coronaria* var. *dwarf* (PI, II); C (I, II) = *T. coronaria* var. *variegata* (PI, II); D (I, II) = *T. coronaria* var. *florepleno* (PI, II) and E I = *T. dichotoma* (PI), P = Population, I-IV = Population number

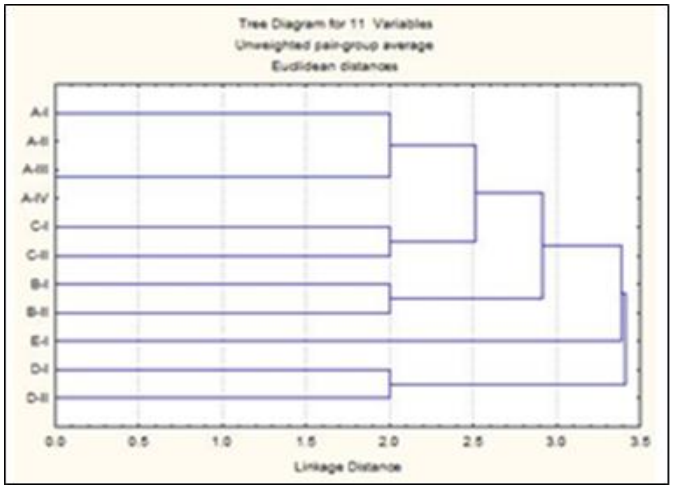

Fig 5: Dendogram obtained from total protein and isoxyme analysis showing relationship among different species, varieties and populations of *Tabernaemontana*. A (I-IV) = *T. coronaria* (PI-PIV); B (I, II) = *T. coronaria* var. *dwarf* (PI, II); C (I, II) = *T. coronaria* var. *variegata* (PI, II); D (I, II) = *T. coronaria* var. *florepleno* (PI, II) and E I = *T. dichotoma* (PI), P = Population, I-IV = Population number

Conclusions

The highest protein content was found in *T. dichotoma* and least in *T. coronaria* var. *dwarf* (population I). Qualitative differences have been observed in one dimension SDS-PAGE profile of the buffer soluble total protein from the leaf tissues of all these species, varieties and populations. The protein profile revealed differences in number of polymorphic bands. The qualitative differences as observed in buffer soluble total protein profile indicated distinct variation among species and varieties of *Tabernaemontana* suggesting differences in genomic constitution at its functional level as the tissues were collected in the same period during their maximum growth in the field. In all the species and varieties studied in the present investigation the number and position of the isozyme activity bands varied. The differential distribution of both esterase and peroxidase activities has revealed genomic diversity among the species and varieties of *Tabernaemontana*. The results of two isozymes in the present study indicated the polymorphisms at the species and variety levels.

Acknowledgment

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